

Effects of Feeding Stearic, Linoleic or Lauric, Acid on Rumen Protozoal Fatty Acid Levels, Proportion of Living Cells and Motility

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Abstract:

Rumen protozoa play an important role in balancing the rumen microbiome along with supporting optimal fiber digestion. Unfortunately, protozoa also excessively degrade protein and stimulate methanogens to increase methane production in dairy cows; supporting protozoal suppression. Lauric and linoleic acid is known to inhibit rumen protozoa when bolus dosed, but the biological reason for this inhibition is unknown. The aim of this study was to determine the impact of these two fatty acids on protozoal intercellular fatty acid levels, proportion of living cells and living fraction motility. The study was conducted using a complete block design in 50-mL tubes fixed with one way gas valves maintained at 39°C. Treatments of lauric (12:0), stearic (18:0) or linoleic (18:2) acid were dissolved in hexanes and dosed at 5, 10, 20 and 40 (μmols) along with a control vehicle without fat. All treatments were adhered to alfalfa substrate (ground to 1mm). Rumen fluid was collected from multiparous cannulated cows and added to tubes containing treatments. Samples and videos were taken at 6 hours post-inoculation. Average cell fluorescence, a proxy for fatty acid concentration using the fluorescent dye Nile Red, proportion of living cells and living fraction motility were measured and standardized as proportion of the control. Stearic acid had no significant impact on measured protozoal parameters. Linoleic acid decreased fluorescence (0.90, 0.84, 0.78, 0.55) linearly ($P = 0.02$), decreased motility (1.03, 1.05, 0.98, 0.88) with a trend towards a linear relationship ($P = 0.07$) and on living cells (0.98, 1.03, 1.03, 0.91) had no significant trend ($P > 0.10$), respectively, with increasing dose. Lauric acid decreased motility (1.02, 0.89, 0.64, and 0.36), living cells (0.99, 0.77, 0.58, 0.32) and fluorescence (0.76, 0.74, 0.73, 0.43), respectively, with increasing dose all in a linear trend ($P < 0.01$). Results show that linoleic and lauric acid adversely affected protozoal health by decreasing proportion of living cells and living fraction motility with increasing fatty acid dose as expected, but also decreased intercellular fatty acid levels with increasing dose. Linoleic acids impacts could be explained by a downstream impact on bacteria or by inhibition of phospholipid formation, whereas increasing lauric acids could result in a disruption in cellular signaling pathways that are important for digestive vacuole maturation thereby killing protozoal cells.

Introduction:

Ciliate protozoa form one of the two groups of eukaryotic microorganisms present in the foregut of ruminant livestock, and they have complex interaction with the rumen microbiome that impact the nutrition of the host organism. Due to the microbial environment in the rumen, ruminants are able to utilize forage based diets that have a high concentration of fiber that would otherwise be less digestible. The presence of protozoa in the rumen supports optimum fiber digestibility and protein degradation the latter of which might explain the former. However, excessive protein degradation and stimulation of methane production by methanogenic archaea are potential reasons to suppress protozoal abundance (Morgavi et al., 2010). Protozoa also contribute to the nutrition of the host animal by the metabolites provided directly in the rumen for bacterial or host use and via the duodenal flow of both metabolites and protozoal cells themselves. However, protozoa have a negative effect of host protein utilization efficiency due to their reduced rate of omasal passage (Jouany, 1996) and via predation on rumen bacteria (Wallace and McPherson, 1987), thus limiting the efficiency of protein reaching the small intestine. A balance between maintaining protozoal health to optimizing fiber digestion and challenging them to decrease methane production and increase protein efficiency is likely needed to maximize animal performance.

Protozoa are often suggested to account for up to half of the microbial biomass of the rumen in dairy cattle, although Firkins et al. (2007) have provided evidence that this value is overestimated by up to half. A main reason for the discrepancy is a result of errors in quantification of cell composition separate from bacterial and feed contamination but also resulting from true differences in cell composition from dietary changes such as supplementation of fat.

Fatty acids are an important nutrient for protozoa, but the research about fatty acid metabolism and usage by protozoa is limited. Lipids are integrated into protozoal cells via de novo lipogenesis, ingestion of dietary material, predation of bacteria, or transmembrane transport or diffusion. However, protozoa prefer to utilize dietary fatty acids rather than synthesize them de novo (Emmanuel, 1974; Demeyer et al., 1978); therefore, dietary lipid composition can influence protozoa. The exogenous supply of the fatty acids lauric and linoleic acid adversely impact protozoal health when bolus-dosed, but due to the limited research in protozoa lipid metabolism the biological reason for this inhibition is unknown.

Linoleic acid (18:2) is an 18 carbon polyunsaturated fatty acid (PUFA) with two unsaturated bonds and is found in vegetable oil. An increase in unsaturation of fatty acids inherently reduces their melting point and allows for them to be more fluid in cellular structures. In an in-vivo study, an inclusion rate of 0.25, 0.5, and 1% resulted in a 48, 88 and 100% decrease in protozoa counts, respectively (Hristov et al., 2004). Rumen protozoa numbers decrease linearly with an increase in the degree of unsaturation of dietary fats (Oldick and Firkins, 2000). Furthermore, protozoa contain a proportional higher concentration of unsaturated fatty acid compared to bacteria (Devillard et al., 2004) and linoleic acid accounts for 8% of total fatty acids in rumen protozoa (Or-Rashid et al., 2007; Sultana et al., 2011). A majority of this and other PUFA are incorporated into protozoal membrane structures; thus, allowing for the fluidity needed for proper membrane integrity (Devillard et al., 2006). PUFA can be converted to monounsaturated and saturated fatty acid via a process called biohydrogenation. In the rumen, up to 70% of PUFA are biohydrogenated to produce conjugated linoleic acid, vaccenic acid (18:1-trans 11), stearic acid (18:0), or various other isomers in low amounts (Wu and Palmquist, 1991).

Lauric acid (12:0) is a 12 carbon saturated medium chain fatty acid that is prevalent in coconut oil. Direct dosing of 12:0 through a rumen cannula in slugs resulted in a greater than 90% decrease in protozoal cell counts (Hristov et al., 2012; Faciola et al., 2013). Lauric acid accounts for less than 0.2% of the total fatty acids in rumen protozoa (Emmanuel, 1974; Or-Rashid et al., 2007; Sultana et al., 2011). Due to the low concentration of lauric acid in protozoa, it is likely that it is involved in a highly conserved biologically pathway where concentration gradients are important.

The objective of this study is to determine the impact of lauric and linoleic acid on rumen protozoal fatty acid concentrations by direct quantification via fluorescent microscopy and protozoal health by measuring proportion of living cells and motility of the living fraction. We hypothesize that lauric, linoleic, and stearic acid will increase intracellular fatty acid concentrations with increasing dose. Second, we expect that lauric and linoleic acid will decrease living cell motility and motility of the living fraction with increasing dose, whereas stearic acid will have no impact.

Methods:

The experiment was conducted using a randomized complete block design with 3 replications. Treatments of stearic acid (18:0), lauric acid (12:0), and linoleic acid (18:2) were dissolved in hexane and dispensed to alfalfa substrate (0.5g/ tube, ground to 1 mm screen size) dosed at 5, 10, 20 and 40 μmol of fatty acid. A fat free control with hexane vehicle was used. After dosing, the hexane was volatilized, thus leaving a dispersion of fatty acids coating the feed. Rumen fluid was collected from cannulated multiparous cows, flocculated for 45 minutes in simplex buffer, and 30 mL was added to each treatment tube. The tubes were then incubated in 50-mL centrifuge tubes fitted with one-way gas valves maintained at 39°C in a hot water bath under anaerobic conditions. At 6 hours of incubation, 50- μL samples were collected from each tube and recorded under a microscope fitted with a camera. Additional samples were collected at the same time and stored in a 5% formalin solution to later be used for intracellular fatty acid analysis.

Nile red, obtained from Fisher Scientific (Pittsburg, PA), was dissolved in acetone (500 $\mu\text{g}/\text{mL}$) and used as adapted from Cole et al. (1990). A dose of 10 μL of Nile red were added to a 1-mL sample to provide 5 $\mu\text{g}/\text{mL}$ final concentration and stained for 30 minutes. The samples were rinsed in 0.9% saline, stained again (5 $\mu\text{g}/\text{mL}$, 30 minutes), and rinsed 3 additional times with 0.9% saline. Cells were then observed using an Olympus BX51 microscope equipped with an Olympus filter-barrier system (Excitation, 510-560 nm; emission, 590nm). Images were quantified using ImageJ to provide average cell fluorescence (Burgess, 2011).

Videos were observed without labels to remove bias. From these videos, proportion of living cells and motility of the living fraction were assessed. The data were measured by scoring each video 0-5 (low-high). Averages were collected. All data for Nile red images and videos were standardized to the fat-free control and displayed as fold change.

Results:

Table 1	micromol fatty acid/0.5 g forage				SE	Probabilities of Contrasts		
	5	10	20	40		Linear	Quadratic	Cubic
Nile Red								
Intensity/Area*								
18:0	1.018	0.813	0.896	0.958	0.069	NS	NS	NS
18:2	0.912	0.860	0.806	0.565	0.079	0.02	NS	NS
12:0	0.786	0.763	0.744	0.440	0.043	< 0.01	0.10	NS
Live Protozoa**								
18:0	1.013	0.983	1.001	1.053	0.055	NS	NS	NS
18:2	0.987	1.038	1.028	0.917	0.032	0.07	NS	NS
12:0	0.988	0.777	0.577	0.317	0.049	<0.01	0.07	NS
Motility Score**								
18:0	1.114	1.099	1.052	1.098	0.050	NS	NS	NS
18:2	1.029	1.044	0.982	0.877	0.083	NS	NS	NS
12:0	1.028	0.889	0.645	0.356	0.071	<0.01	NS	NS

Table 1: Fold changes of fluorescent staining (proxy for fatty acid concentration), living cell proportion and motility of living fraction of mixed rumen protozoa after 6 h of incubation with stearic (18:0), linoleic (18:2) and lauric (12:0) acid adsorbed to ground alfalfa (ground to 1mm). All data were collected at 6 h of incubation and normalized to control (fat-free hexane vehicle). *Fatty acid source, fatty acid concentration interaction (**P** = 0.02). **Fatty acid source, fatty acid concentration interaction (**P** < 0.01). NS: not significant (**P** > 0.10). SE: standard error.

Stearic acid had no significant impact on intercellular fatty acid concentration, proportion of living cells or living cell motility ($P > 0.10$). Linoleic acid decreased cell fluorescence linearly ($P < 0.01$) and had a trend towards a negative linear ($P = 0.07$) relationship for proportion of living cells, but there was minimal impact when 40 μmol of linoleic acid was dosed. Lauric acid decreased cell fluorescence linearly ($P < 0.01$) and had a quadratic trend ($P = 0.10$), decreased proportion of living cells linearly ($P < 0.01$) with a trend towards quadratic ($P = 0.07$), and decreased motility linearly ($P < 0.01$).

Conclusion:

The decrease in cellular lipids differs from expectations. Linoleic acid likely either inhibited biohydrogenation pathways or phospholipid formation, which decreased fatty acid levels but still allowed for the viability of protozoal cells. For lauric acid, it can likely be attributed to the high mortality rate. When cells die, their cell membrane integrity is lost, allowing contents to leak out. The high mortality rate is likely to occur from a competitive type of inhibition due to the large influx of lauric acid during bolus dosing.

The decrease in intercellular lipid levels from linoleic dosing could be explained by a downstream impact on bacteria. Biohydrogenation primarily conducted by bacteria (Harfoot and Hazlewood, 1997), so an influx of unsaturated fatty acid into the rumen environment could overwhelm biohydrogenating bacteria and therefore inhibit them. Smaller protozoa obtain a large proportion of their energy from bacteria as well as starch (Diaz et al., 2014). Biohydrogenating bacteria associate with the particulate phase (Jenkins et al., 2008), so a decrease in particulate bacteria and therefore a decrease in bacterial engulfment by protozoa could adversely impact those that depend on bacteria as a nutrient source.

Nile red is fluorescent in hydrophobic regions (Cole et al., 1990); structures seen under red fluorescence include phospholipids, amphipathic lipids and strongly hydrophobic proteins (Fowler and Greenspan, 1985). Mixed protozoa contain a high proportion of their lipids as phospholipids (85.5%; Harfoot, 1978), so it is likely that a majority of cellular lipids would be measurable with the Nile red assay we used. It is possible that linoleic acid results in an inhibition of phospholipids formation; thus, explaining the decreased Nile red fluorescent intensity and relatively minimal impact on proportion of living cells and living fraction motility.

If the level of lauric acid is increased in protozoal cells, it has the potential to disrupt cellular function either by disrupting membrane integrity or an unknown metabolic process. Lauric acid inhibition of protozoa cells could be attributed to a competitive type of inhibition. The low levels of intracellular lauric acid (Emmanuel, 1974; Or-Rashid et al., 2007; Sultana et al., 2011) and the rapid influx needed to inhibit a large portion of the protozoal fraction (Hristov et al., 2012; Faciola et al., 2013) help explain this. The predominant fatty acid in eukaryotic phosphoinositides, the cell signaling molecule, is myristic acid (14:0) (Leondaritis and Galanopoulou, 2000). Cell signaling is a highly conserved process in eukaryotic cells (Leondaritis et al., 2011), so an influx of lauric acid could competitively displace myristic acid and thereby disrupt the ability of phosphoinositides (PI) to anchor membranes in their normal cell signaling pathways, jeopardizing cell function. The specific enzyme of concern is phosphoinositol-3-kinase (PI3K). PI3K is necessary for the homeostasis and signaling of various inositides, one of which signals for digestive vacuole maturation, and for formation of IP_3 (inositol 3-phosphate), which is critical for intracellular Ca^{++} homeostasis in eukaryotic cells (Diaz et al., 2014).

Future studies that directly explore the interaction between cell signaling pathways for linoleic and lauric acid need to be conducted. Phagocytosis has been linked with digestive vacuole formation (William et al., 1986). Protozoa engulf bacteria at an increasing rate as the bacteria population is increased (Williams and Coleman, 1992). With the use of fluorescent latex beads following a method previous used in our lab (Diaz et al., 2014) we can quantify the rate of bacterial phagocytosis to determine if a correlation is present between linoleic or lauric acid and digestive vacuole formation. If digestive vacuole maturation is blocked by either fatty acid, it would be expected that bead uptake would be inhibited and an increase in protozoal chemotaxis towards other nutrients, such as glucose, would occur.

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